Methanol and isopropanol embryo dosage response curves for wild-type and ethanol-sensitive zebrafish.

Presented by Ashley Anderson

Johann Eberhart, PhD
Supervising Professor

5/13/2016
Date

Ruth Buskirk, PhD
Honors Advisor in Biology

Date

Ruth

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Ashley Anderson, Ben Lovely, Dr. Johann Eberhart
Department of Molecular Biosciences; The University of Texas at Austin; Austin, TX USA

Abstract

It is well established that ethanol has an array of negative effects on developing embryos, from craniofacial abnormalities to cognitive deficits and behavioral disorders. Fetal Alcohol Spectrum Disorders (FASD) describes this phenotypic spectrum caused by embryonic ethanol exposure. However, the effects of other small alcohols, such as methanol and isopropanol, have on development are poorly understood. Multiple factors can contribute to the teratogenicity of small alcohols, including timing, dosage and genetic background. Zebrafish (*Danio rerio*) has been shown to be a powerful model in the study ethanol teratogenesis and can serve as a model to study methanol and isopropanol teratogenicity. Here we provide evidence of the dose response to methanol and isopropanol in a wild type and an ethanol-sensitive mutant zebrafish line. We determine the lethal concentrations of methanol and isopropanol on wild type and ethanol-sensitive mutants. We also show effective dose that leads to malformations of the craniofacial skeleton, including defects to the lower jaw and palate. Our data suggest that ethanol-sensitivity may predict sensitivity to other small alcohols. Overall, our results begin to characterize the effects of methanol and isopropanol on developing embryos.

Introduction

The effects of ethanol on the developing human fetus have been shown to cause developmental defects ranging from general growth retardation to craniofacial abnormalities and cognitive disorders. Described as Fetal Alcohol Spectrum Disorders (FASD), about 1% of the United States population is affected by some form of FASD which costs the United States up to $5.5 billion a year (Centers for Disease Control and Prevention). The small carbon alcohols, methanol and isopropanol, are equally as prevalent ethanol yet we know little of the effects of these alcohols on development.

Methanol and Isopropanol Exposure

There are many routes of methanol exposure. Illegally produced alcoholic beverages commonly contain methanol, often in the form of “wood grain alcohol” (TOXNET). Methanol occurs naturally in low concentrations in most conventionally and legally produced alcohols, but the higher doses found in illegally produced alcohols can be lethal to humans (Paine et al. 2001). Methanol amounts as low as 50 mL can be lethal to a fully grown adult (TOXNET). The highest concentration to safely consume has been shown to be 4% in a beverage, although for safety reasons it is not recommended to consume alcohol that contain over 0.4% methanol (Paine and Dayan 2001). The highest risk of methanol exposure involves persons who come in contact with methanol’s industrial applications. Methanol is one of the top five most widely used solvents around the world as it can be used in making plastics, building materials, car

parts, paints, in various forms of energy production or as fuel for transportation, among many other uses (Methanol Applications).

Methanol is often found in fermented fruits and vegetables along with wine and beer. There exists a natural level of methanol in the body due to diet and as a result of metabolic processes in the body. Small amounts of methanol can be excreted by the lung and kidneys, but the majority of methanol is processed in the liver via alcohol dehydrogenases into formaldehyde, then formic acid and carbon dioxide (TOXNET). In all species that do not readily metabolize formic acid, metabolic acidosis can cause death. Primates and humans do not metabolize formic acid and demonstrate an increase in formic acid in the blood following methanol ingestion (TOXNET). Even in species that can metabolize formic acid, depression of the central nervous system can lead to coma and death if too much is ingested (TOXNET). For example, rodents can metabolize formic acid and do not show an increase in blood formic acid after methanol exposure, but methanol causes craniofacial, axial skeleton, and central nervous system defects in both mice and rat development (Degitz et al. 2004). Most case study evidence is found concerning only acute methanol exposure in humans, whether from direct ingestion, inhalation of fumes, or absorption through the skin. The process of removing methanol from the body through the lungs, the kidneys, and the liver, is relatively slow compared to the body's ability to remove ethanol using these three similar removal mechanisms (TOXNET). In a study of maternal-fetal pharmacokinetics of methanol in rats, fetal liver detoxification was shown to be less than 10% that of the adult liver metabolism (Pollack 1996). The relatively low amount of methanol required for lethality in adults combined with the abundance of daily methanol exposures and the lower processing capabilities in embryos leads to the question of how methanol exposure might affect fetal development.

Isopropanol is considered to be less toxic than methanol and is one of the most common disinfectants, widely used in hospitals, laboratories, and other sterile environments. Isopropanol is most commonly found in households as rubbing alcohol, hand sanitizer, or pre-soaked disinfectant pads. In addition, it can be found in perfumes, body lotions, antifreeze, essential oils, cosmetics, some inks, and in pharmaceutical aids (TOXNET). The group at highest risk of isopropanol toxicity in the household are children, who might ingest these products. Also workers, in the production of these various chemicals, are at high risk of toxic isopropanol exposure. Anywhere from 20 to 50% of isopropanol that is absorbed gets excreted through the lungs or kidneys. The remaining isopropanol is processed via alcohol dehydrogenase in the liver to acetone, then to formic acid and carbon dioxide (TOXNET). The lethal dose of isopropanol ingestion is about 250 mL, or 5 times that of methanol, although case study evidence indicates that acute doses of about 100 mL have caused death (TOXNET). While less commonly consumed than methanol, it is of interest to explore the effects of propanol on embryonic development due to the high relative exposure and abundance of isopropanol.

Methanol and Isopropanol in Development

Previous studies indicate that methanol and isopropanol are teratogenic. Methanol has been shown to cause neural tube defects, holoprosencephaly, facial dysgenesis, growth retardation, increased embryo death, and cervical vertebral malformations in in several model

systems (Bolon et al. 1994, Degitz et al. 2004, Rogers et. al 1997, Rogers et al. 2004, Mellerick et al. 2004, Zhang and Rawson 1995). In *Drosophila* embryos, methanol exposure caused altered cell movements associated with gastrulation and cell band movements, ventral-midline defects, and apoptosis in parts of the central nerve system (Mellerick et al. 2004). Mice embryos were shown to develop exencephaly when the mother was exposed to high concentrations of methanol on gestational day 8 (Dorman et. al 1995). Methanol negatively affects neurons and ganglia in the brain in mice (Degitz et al. 2004). Gastrulation-stage methanol exposure in mice causes death in migrating neural crest cells and affects the anterior mesoderm and neuroepithelium (Degitz et al. 2004). The craniofacial defects seen were micro/anophthalmia, holoprosencephaly, facial clefts and gross facial angenesis (Rogers et al. 2004). A study of cultured rat and mouse embryos exposed to methanol indicates that cell death is responsible for cranial abnormalities and in the eye, ear, and cleft palate (Abott et al. 1995). In a study of methanol exposure during gastrulation in mice, the craniofacial malformations resulting from methanol indicated that methanol and ethanol may have common targets and common modes of action (Rogers et al. 2004).

Though isopropanol is considered less toxic than methanol, it can be teratogenic as well. When pregnant rats were exposed to 10,000 ppm isopropanol vapors, the dams experienced narcosis, reduced overall weight gain, and were more likely to experience resorption (Nelson 1988). The pups demonstrated a range of malformations to the axial skeleton and limb as well as cardiovascular and urinary defects (Nelson et al. 1988). In mice, isopropanol causes decreased average litter weight, a range of skeletal defects (Faber et al. 2008). Outside of these studies, very little is known about isopropanol teratogenicity. Studying the effects of varying concentrations of methanol and isopropanol on the developing zebrafish embryo leads to more knowledge on the effects of small alcohols during development.

Zebrafish as a model organism for small alcohol teratogenicity

Zebrafish is an excellent model for examining small alcohol teratogenicity. They are genetically tractable, externally fertilized, are embryonically transparent, have high fecundity and a rapid development time (Bilotta et al. 2004). The zebrafish stages of development have been well characterized allowing for precise knowledge of the processes occurring at each stage of development. The embryonic transparency allows for in depth visualization of these developmental processes. Furthermore, experiments focusing on zebrafish exposure to ethanol have established several physical abnormalities that can occur, depending upon the timing and dosage of the ethanol exposure as well as the genetic background of the exposed embryo.

Ethanol has been previously shown to be able to pass through the chorion and reach the developing embryo (Bilotta et al. 2004, Lovely et al. 2014). It has been found that other small alcohols, such as methanol and isopropanol, similarly can pass through the chorion and are present in the embryonic tissue (Hutchinson et. al 2006). Zebrafish exposed to ethanol are often edemic with smaller than average eyes and an increase in the inter-eye distance. In addition, they can exhibit defects in development of the heart, ear and craniofacial skeleton.

Here we show that zebrafish can be used to demonstrate the effects of methanol and isopropanol. We perform a dose dependent response on embryo lethality and the on craniofacial development. We show that methanol exposure has very little effect on embryos survival and craniofacial development. We then show that isopropanol can result in embryo death above 1% in wild type embryos and above .75% in mutant embryos. Lower doses can lead to very stereotyped craniofacial malformations of the lower jaw and palatal skeleton.

Materials and Methods

Zebrafish (Danio rerio) care and use and alcohol exposure regimen

Two strains were utilized in this study, *Tg(fli1:EGFP)*^{y1} transgenic embryos (Lawson and Weinstein, 2002, *fli1* in the text) and *bmp4*^{st72} (Stickney et al., 2007, *bmp4* in the text). The solutions were mixed immediately before application to the embryos to reduce evaporation of these volatile small alcohols over time. Embryos were treated with methanol or isopropanol starting at the 6 hours post-fertilization (hfp). Both methanol and propanol were treated in .1%, .25%, .5%, 1%, 1.5%, and 2% doses in the first clutch of *fli1:EGFP* embryos. After noting the response, the isopropanol dosage was changed to .05%, .1%, .25%, .5%, .75%, and 1% for future experiments. The *fli1:EGFP* embryos were tested in groups of 30, and the *bmp4* embryos were tested in groups of 50. Embryos were then observed over five days to determine the number that died each day, to note the phenotypes, and to maintain the plate of developing zebrafish. LC50 and EC50 scores were determined for each dose in both backgrounds.

Bone and cartilage labeling

Embryos at 5 days post-fertilization (dpf) were stained with Alcian Blue labeling cartilage and Alizarin Red labeling bone. Embryos were fixed in 2% PFA/1X PBS at room temperature, nutating for 1 hour. The embryos then nutated in 1 mL of Alcian blue overnight. The embryos were destained and rehydrated using sequential ethanol and Tris solutions for five minute durations. The first was 80% ethanol, 100 mM Tris at pH 7.5, and 10 mM $MgCl_2$. The second was a 50% ethanol, 100 mM Tris at pH 7.5 solution. The third was a 25% ethanol, 100 mM Tris at pH 7.5 solution. The embryos were then bleached for 10 minutes with 1 mL 3% H_2O_2 and .5% KOH solution. Next, a 25% glycerol and .1% KOH solution was used for two ten-minute washes. Next, 1 mL of Alizarin red was applied and the embryos were nutated for 30 minutes. The embryos were destained with 50% glycerol and .1% KOH solutions for 10 minutes. This was replaced with fresh 50% glycerol and .1% KOH solution and stored at 4 ^{o}C.

Images

Images of cartilage stains were collected on a Zeiss Axioimager (Zeiss). Images were processed in Microsoft Powerpoint (Microsoft).

Results

Exposure of wild type embryos to methanol and isopropanol

Control and methanol-treated *fli1* embryos over two different experiments showed an initial die-off between day 0 and day 1 (Figures 1-3). The 2% methanol solution was the only dose that reached an LC50, though the survival rates of .5% and 1.5% methanol solutions are just under 60% (Figure 1). When split between two experimental runs the, the 2% methanol

solution experienced the most embryo death in clutch #1 (Figure 2) while the .5% methanol solution had the most embryo death in clutch #2 (Figure 3). The results suggest that *fli1* embryos reach an LC50 when treated with 2% methanol. Overall the results were highly variable, demonstrating that repetition is necessary to determine the survival trends and experimental artifacts that could cause embryo death.

Control and isopropanol-treated *fli1* embryos over two different experiments showed an initial die-off between day 0 and day 1 (Figures 4-5). The treatment of clutch #1 showed that 1.5% and 2% isopropanol solutions were completely lethal with only embryos treated with .5% isopropanol reaching an LC50 (Figure 4). For the second clutch, the maximum tested dose was lowered to 1% (Figure 5). In clutch #2, .1% isopropanol experienced the most die-off at 50% survival, but the .05% and 1% isopropanol were close with 53% survival (Figure 5). This suggests further exploration of the doses between .1% and 1.5% in future experiments will be required to determine the LC50 of isopropanol. Overall, the analysis of embryo survival demonstrated that the doses of both methanol and isopropanol used on wild type fish did not result in large numbers of embryonic death.

With large numbers of embryos surviving the range of doses of both alcohols we were able to determine the effects of methanol and isopropanol on zebrafish development. In general, edema occurred in *fli1* embryos in solutions at 1% methanol concentration and higher, as well as in .5% isopropanol solutions and higher. There were no overt craniofacial defects present in solutions containing methanol at any percentage. However, isopropanol solutions did cause craniofacial defects at .5%, .75%, and 1% concentrations. The 1% isopropanol solution showed multiple embryos with both unilateral loss of the Meckel's cartilage as well as misshapen Meckel's, ceratohyal and ceratobranchial cartilages (Figure 6B compared to A). In addition, broken trabeculae were observed in a portion of the clutch (Figure 6D compared to C). The penetrance and expressivity of malformations decreased as the percentage of isopropanol decreased (data not shown). Therefore, the dose ranges used on wild type fish can be used on mutant lines to determine the impact of the gene-alcohol interactions on development, in particular craniofacial development.

Treatment of bmp4 embryos with methanol and isopropanol

Control and methanol-treated *bmp4* embryos showed initial die-off between day 0 and 1 as observed in *fli1* embryos (Figure 7 compared to Figure 1). The .5% methanol solution experienced the most die-off and reached survival rate of 54%, although the 2% methanol solution resulted in the second-most die-off with a survival rate of 58% (Figure 7). An LC50 for methanol-treated *bmp4* was not found in this single experiment, therefore this experiment must be repeated, perhaps including higher doses of methanol, in order to determine the survival trends and experimental artifacts that cause embryo death.

Control and isopropanol-treated *bmp4* showed initial die-off between day 0 and 1 as seen in *fli1* embryos (Figure 8 compared to Figure 4). However, the 1% isopropanol solution resulted in complete die-off by 5 dpf (Figure 8). No other solution reached an LC50, but the .75% isopropanol solution resulted in survival rates at of 58% and the .5% at 60% (Figure 8). An

LC50 for isopropanol-treated *bmp4* was not found in this single experiment, implying that it should be repeated, perhaps with isopropanol solutions between 1% and 1.5%, in order to determine the survival trends and rule out any experimental artifacts that could cause embryo death.

The survival rates in these dose response curves suggest that we might have exposed *bmp4* mutant embryos to doses of methanol and isopropanol that result in development defects. As in *fli1* embryos, edema occurred in solutions at 1% methanol concentration and higher, as well as in .5% isopropanol solutions and higher. There were not craniofacial defects present in any *bmp4* embryos in any percentage of methanol. Isopropanol solutions caused craniofacial defects at .5% and .75% concentrations. The .75% isopropanol solution showed multiple embryos with Misshapen Meckel's, ceratohyal and ceratobranchials cartilages (Figure 9B compared to A), as well as broken trabecula (Figure 9D compared to C). Again, penetrance and expressivity of malformations decreased in the .5% isopropanol solution (data not shown). These results suggest that genetic background may play a role in sensitivity to isopropanol teratogenesis.

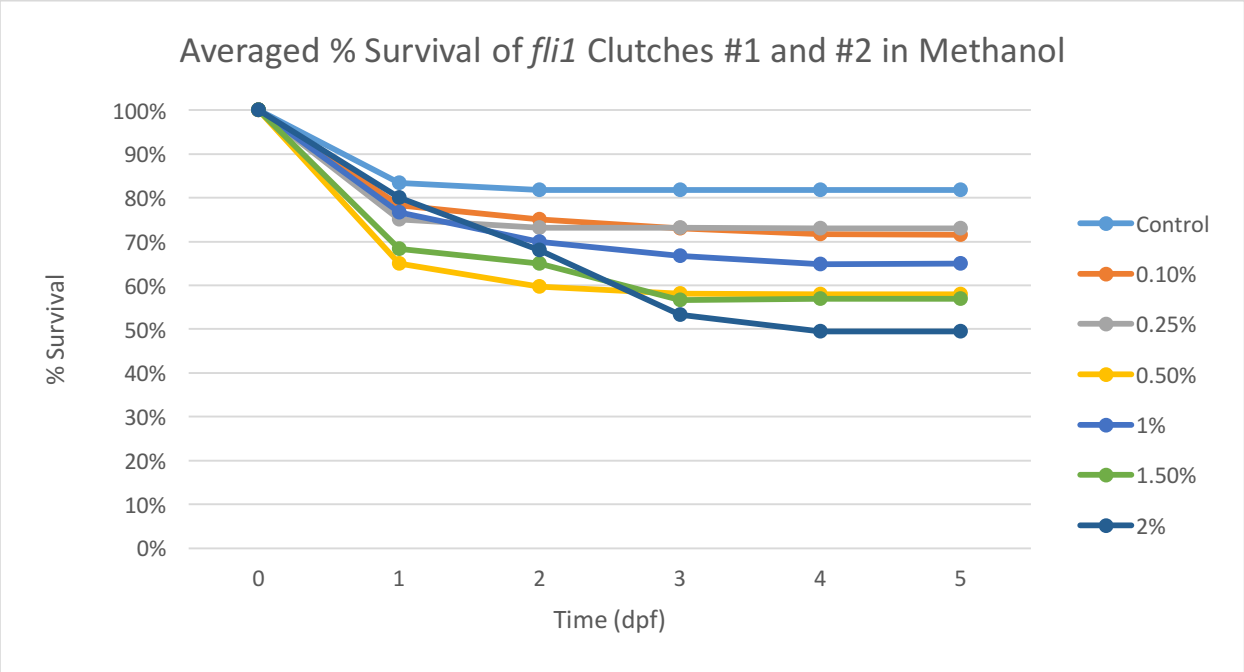

Figure 1. The averaged % survival over 5 days of the 60 total *fli1* embryos from the first and second clutches. These embryos were dosed with the listed methanol concentrations at 6 hpf. The 2% methanol solution demonstrated the largest difference in survival from the control in the averaged % survival. The 1.5% and 0.5% methanol concentrations demonstrated the second largest difference from the control.

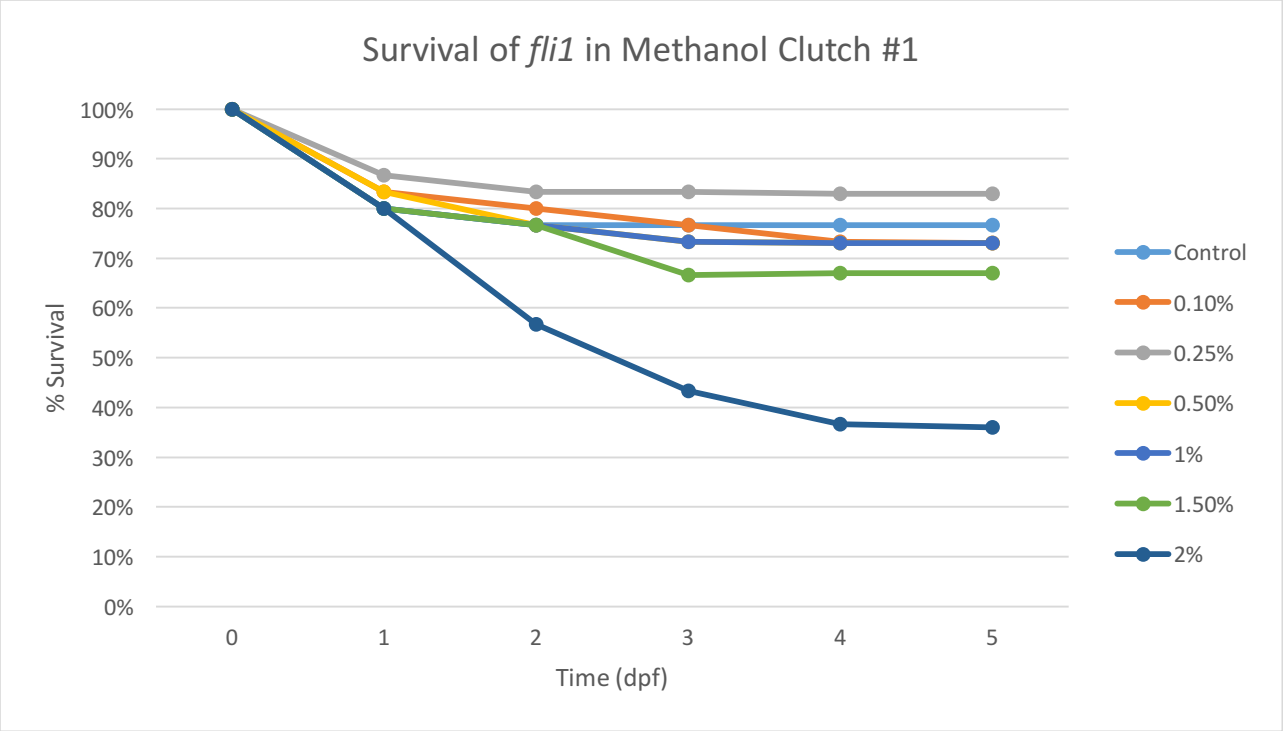

Figure 2. Percentage of surviving embryos over 5 days of the first clutch of 30 *fli1* embryos. These embryos were dosed with the listed methanol concentrations ranging from 0% - 2% at 6 hpf. Only 2% shows a difference in survival from the control.

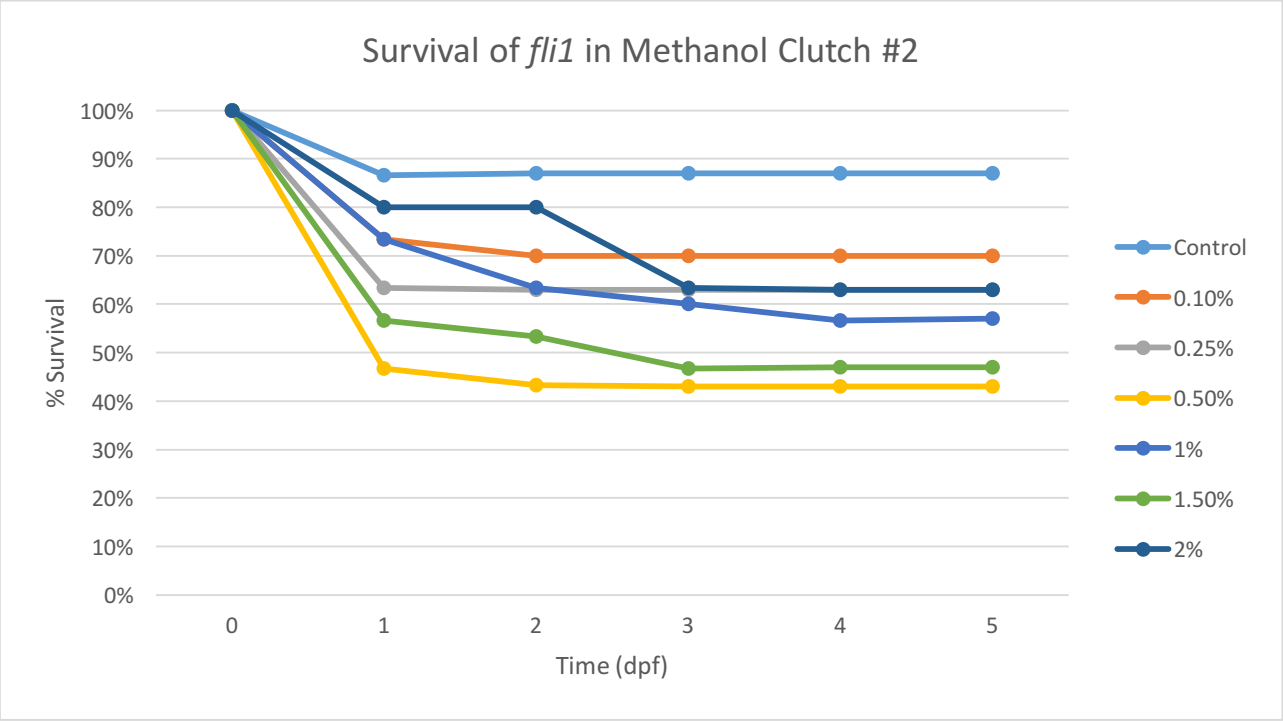

Figure 3. Percentage of surviving embryos over 5 days of the second clutch of 30 *fli1* embryos. These embryos were dosed with the listed methanol concentrations ranging from 0% - 2% at 6 hpf. The 0.5% and 1.5% demonstrate the largest difference in survival from the control.

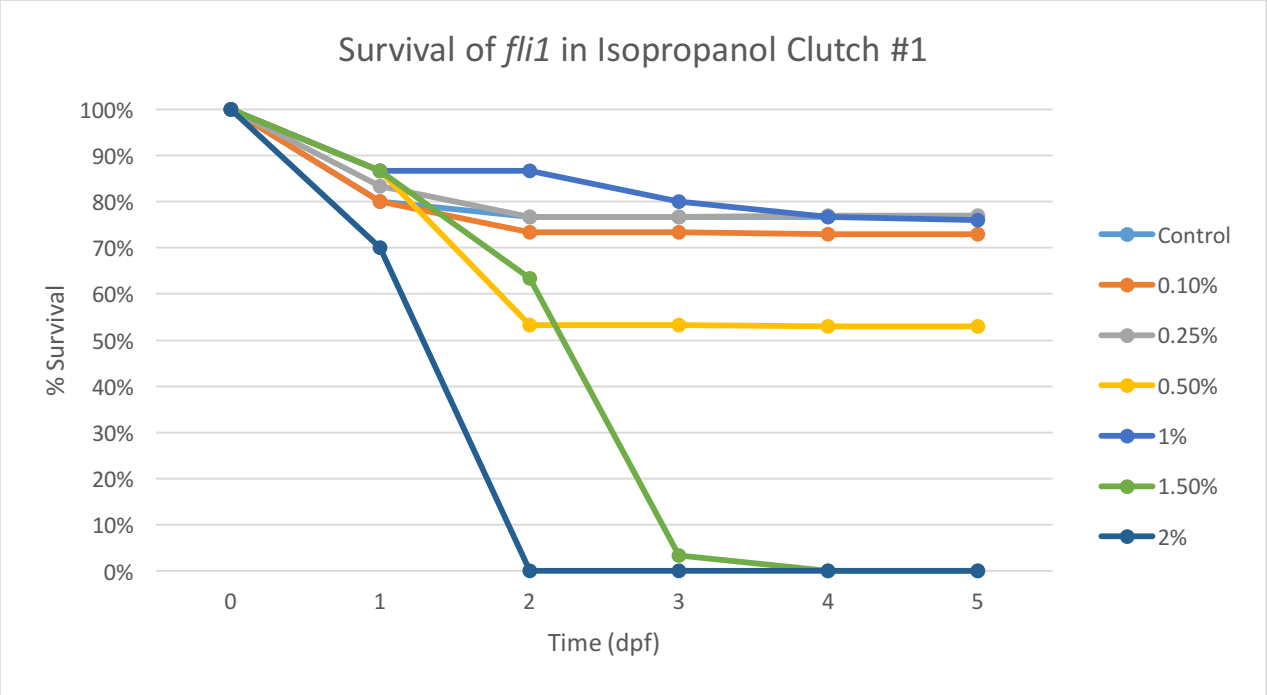

Figure 4. Percentage of surviving embryos over 5 days of the first clutch of 30 *fli1* embryos. These embryos were dosed with the listed Isopropanol concentrations ranging from 0% - 2% at 6 hpf. The 1.5% and 2% isopropanol concentration were completely lethal. The .5% isopropanol demonstrated the largest decrease in survival from the control. The 1% isopropanol demonstrated similar survival to the .1% and .25% concentrations, all of which showed similar survival as the control.

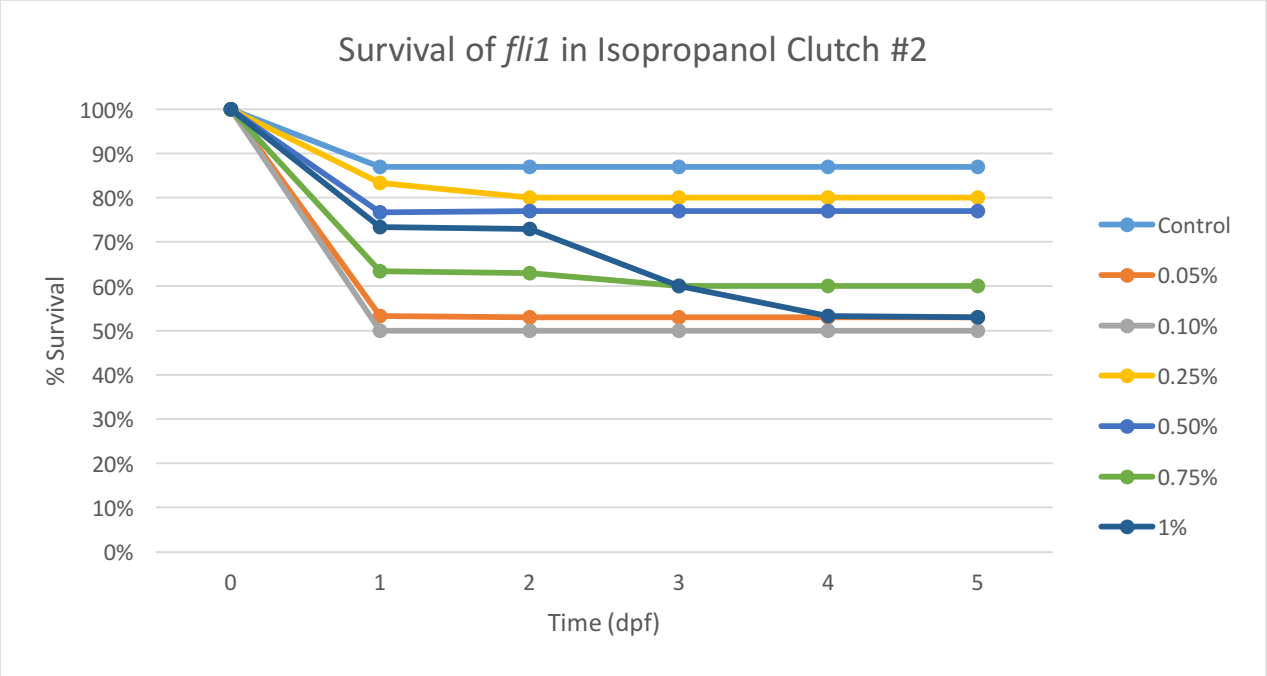

Figure 5. Percentage of surviving embryos over 5 days of the second clutch of 30 *fli1* embryos. These embryos were dosed with the listed isopropanol concentrations ranging from 0% - 1% at 6 hpf. The.75%, .05%, 1.0%, and 0.1% isopropanol concentrations demonstrated the largest difference % survival from the control in that order. The .25% and .5% isopropanol concentrations demonstrated the least difference from the control.

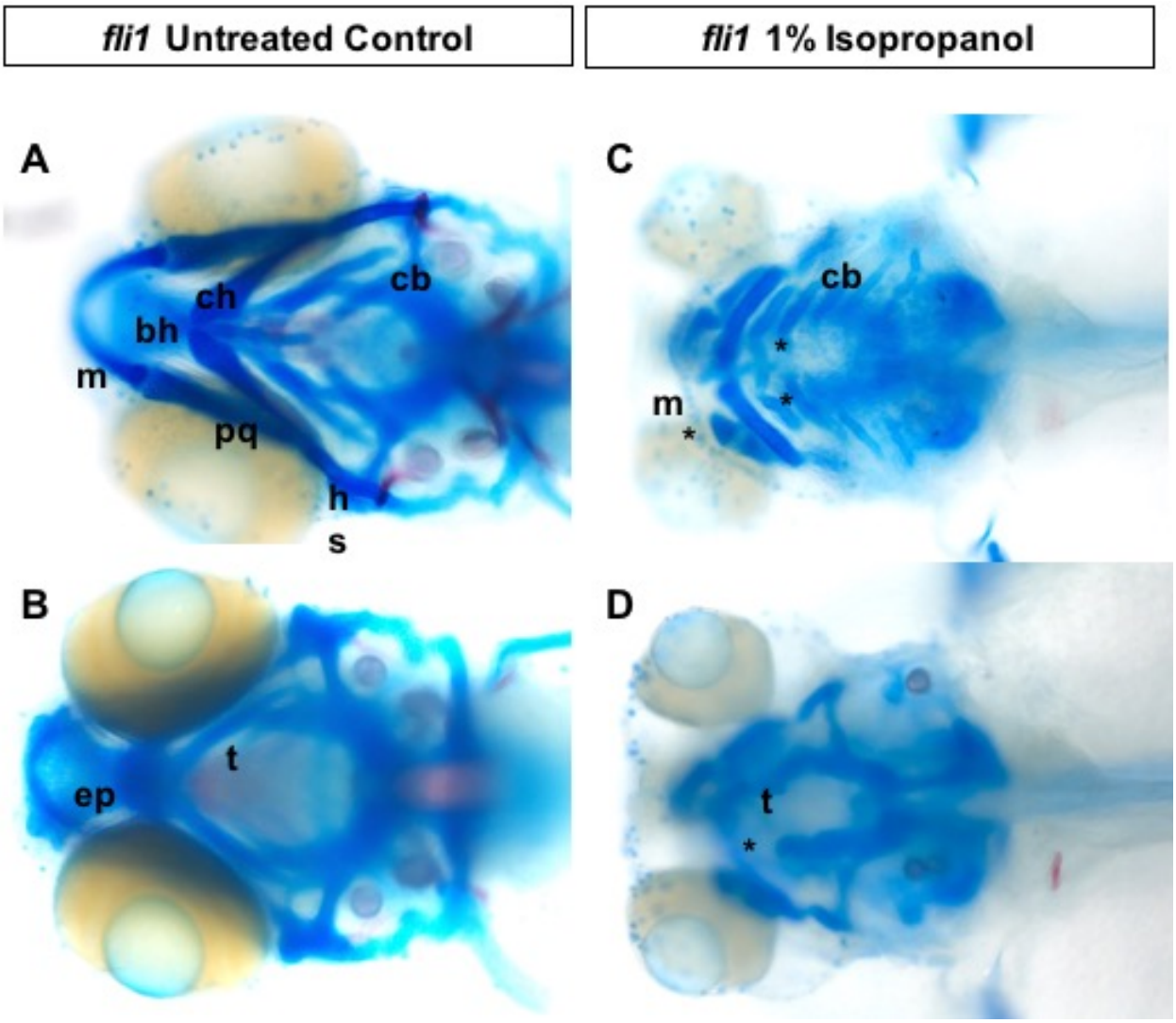

Figure 6. Whole mount images of the 5 dpf zebrafish craniofacial skeleton labeling cartilage in blue and bone in red. In all images, anterior is to the left with asterisks (*) denoting craniofacial defects. **A:** Vental view of an untreated *fli1* control viscerocranium. **B:** Dorsal view of the *fli1* control neurocranium. **C:** Ventral view of a *fli1* viscerocranium of an embryo exposed to 1% isopropanol. There is a missing Meckel's and misshapen ceratobranchials. **D:** Dorsal view of a treated *fli1* embryo shows that there are breaks in the trabeculae. Labels legend: m, Meckel's; bh, basihyal; ch, ceratohyal; pq, palatoquadrate; hs, hyosympletic; cb, ceratobranchial; ep, ethmoid plate.

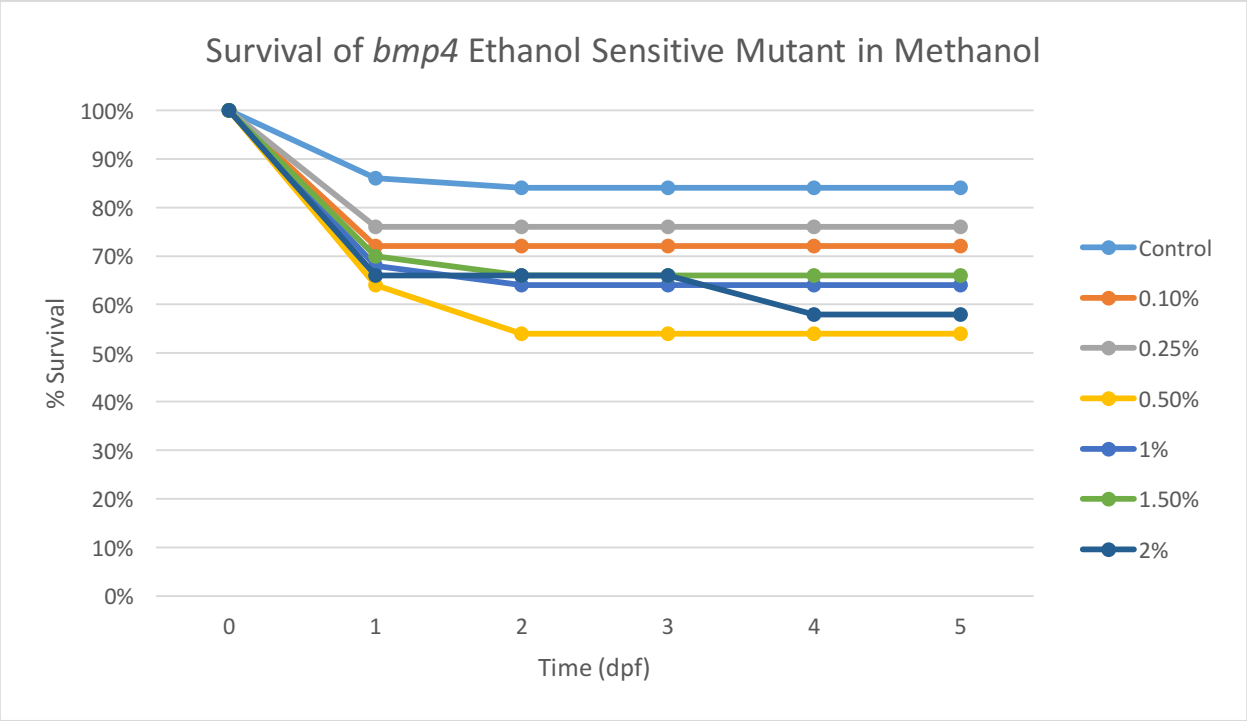

Figure 7. Percentage of surviving embryos over 5 days of the first clutch of 50 *bmp4* embryos. These embryos were dosed with the listed methanol concentrations ranging from 0% - 2% at 6 hpf. The 0.50% methanol concentration demonstrates the largest difference from the control, followed by the 2%, 1%, and 1.5% methanol concentrations. The 0.25% and 0.10% methanol concentrations demonstrated the least difference from the control.

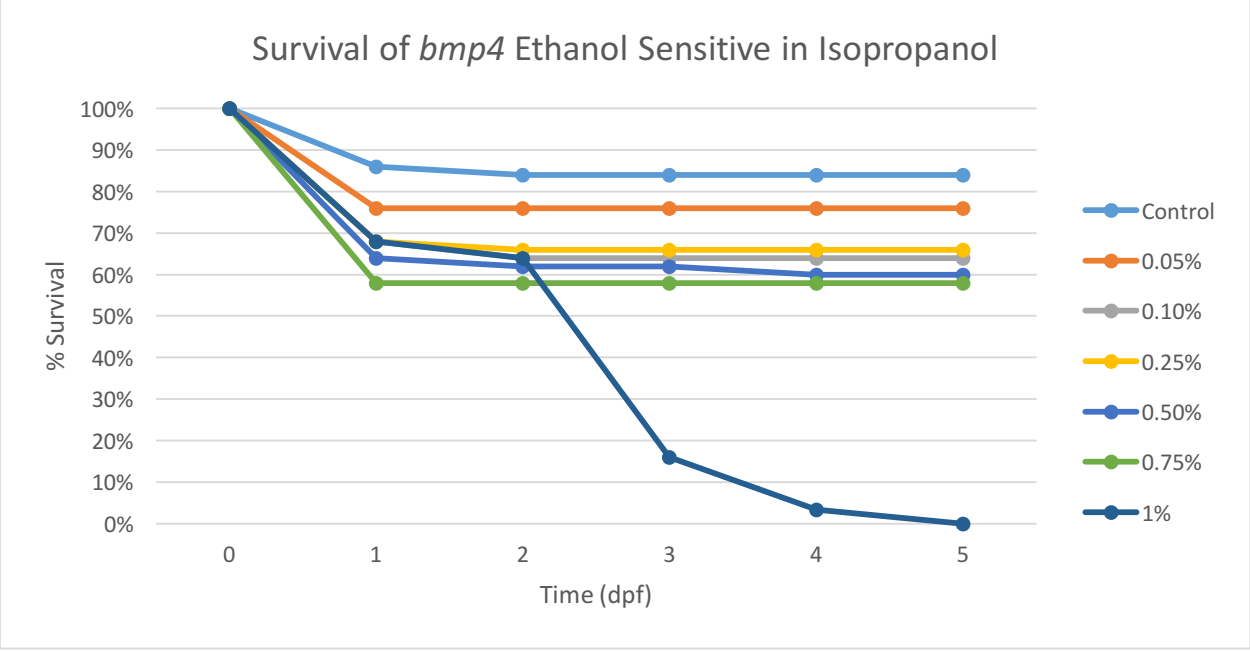

Figure 8. Percentage of surviving embryos over 5 days after the clutch of 50 *bmp4* embryos were dosed with the above isopropanol concentrations at 6 hpf. The 1% isopropanol concentration demonstrated complete lethality. The 0.75%, 0.50%, 0.10%, and 0.25%

isopropanol concentrations demonstrated the second most difference in % survival from the control. The 0.05% isopropanol concentration demonstrated the least difference in % survival from the control.

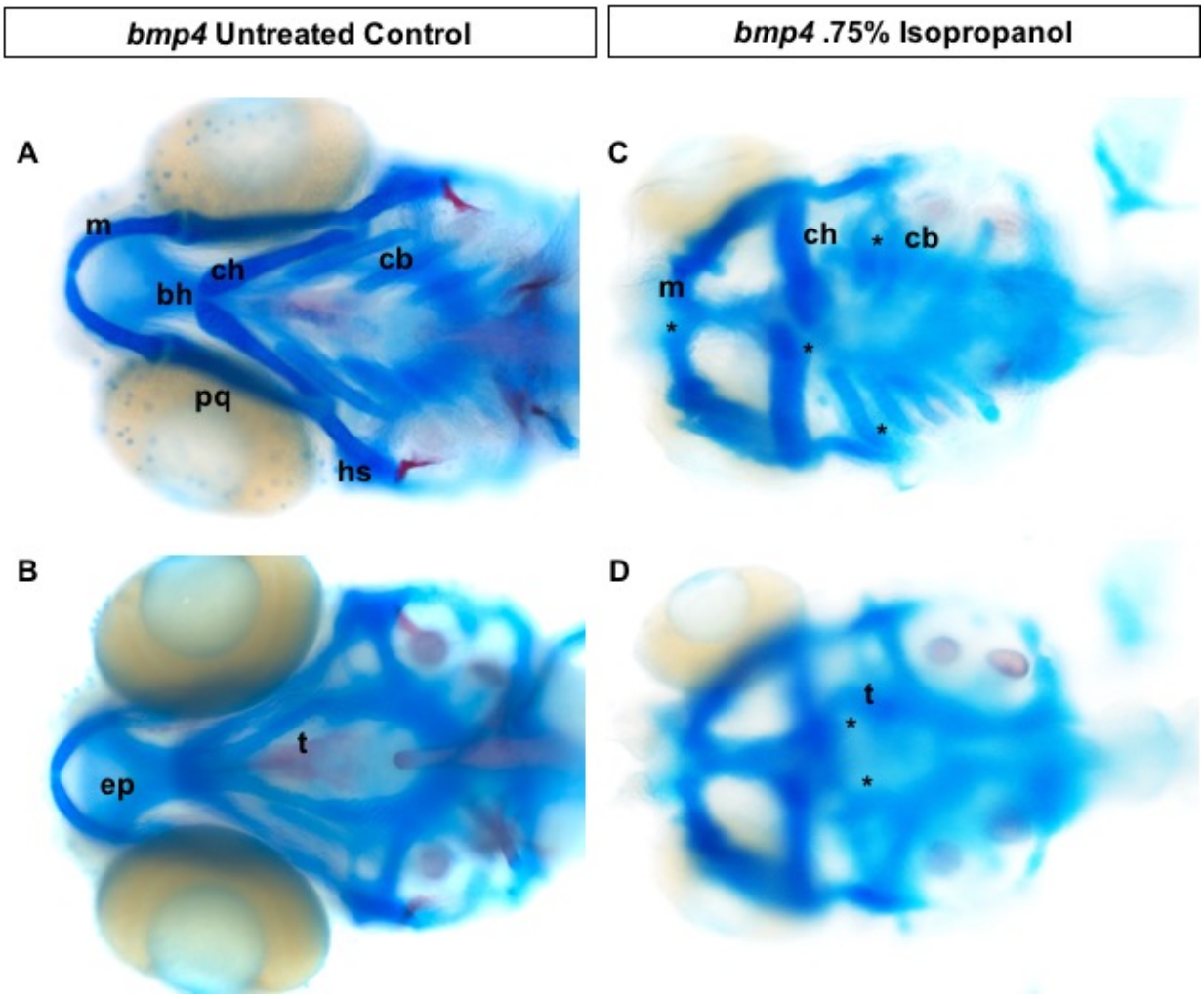

Figure 9. Whole mount images of the 5 dpf zebrafish craniofacial skeleton labeling cartilage in blue and bone in red. In all images, anterior is to the left with asterisks (*) denoting craniofacial defects. **A:** Vental view of an untreated *bmp4* control viscerocranium. **B:** Dorsal view of the *bmp4* control neurocranium. **C:** Ventral view of a *bmp4* viscerocranium of an embryo exposed to .75% isopropanol. There is a missing Meckel's and misshapen ceratobranchials. **D:** Dorsal view of a treated *bmp4* embryo shows that there are breaks in the trabeculae. Labels legend: m, Meckel's; bh, basihyal; ch, ceratohyal; pq, palatoquadrate; hs, hyosympletic; cb, ceratobranchial; ep, ethmoid plate.

Discussion

Here we show that zebrafish can be used to study the developmental impacts of methanol and isopropanol. Our dose response studies show that methanol exposure has very little effect on embryos' survival rates and craniofacial development. Conversely, isopropanol can result in embryo death above 1% in *fli1* wild type embryos and above .75% in *bmp4* mutant

embryos. Doses at 1% and .75% in *fli1* and *bmp4* embryos, respectively, can lead to craniofacial malformations of the lower jaw and palatal skeleton, highly similar to ethanol treated embryos. This leads us to believe that the effects of ethanol and isopropanol on embryo development are similar. Further experimentation should be done in order to determine how these defects occur. It could be that the cellular signaling mechanisms in response to ethanol and isopropanol are similar, and further experimentation could determine these mechanisms.

It has been shown that ethanol can result in different phenotypes in different backgrounds of zebrafish while tissue concentrations remain similar (Bilotta et al. 2004). There are not only phenotypic differences, but also different levels of toxicity. How these differences between the genetic backgrounds lead to ethanol sensitivity remain poorly understood (Lovely et al. 2014). This raises the question of how other teratogens, such as methanol and isopropanol impact embryos of differing genetic backgrounds. In future experiments, the background data should be gathered for all wild type backgrounds so that any analyses of mutant lines will be well controlled. In our hands, we had issues with our wild type AB line laying eggs and had to move to the next closest background compared to the AB line, the *fli1* line. In the future, these experiments must be completed in the AB line in order to draw conclusions about the comparison between lethality and effectiveness in the mutant *bmp4* line which is in the AB background.

The differing results between isopropanol and methanol suggests that something about the alcohols or the metabolic intermediates lead to toxicity in zebrafish. In humans, ethanol is processed into acetaldehyde then to acetic acid where it enters the Kreb's cycle. Methanol is broken down into formaldehyde and then formic acid. Isopropanol is broken down into acetone and then acetate. The ability of zebrafish to metabolize methanol and isopropanol is poorly understood. It is of interest to pursue the mechanisms of metabolism of methanol and isopropanol to examine how methanol is tolerated at higher concentrations than isopropanol in developing zebrafish embryos.

The initial die-off could be due to a number of reasons such as contaminants in the plates, failure to remove unfertilized eggs or improper handling of the embryos. Subsequently, the variation in our results indicates that the experiments need to be repeated to eliminate any potential confounds in our experimental methodology. An additional concern is how the volatility of both alcohols may impact our final solution concentrations. Because these are volatile small alcohols, the composition of the solutions could have changed throughout the five-day testing period as the alcohols evaporated and condensed onto the bottom of the plate's lid in the incubator. In the future, the composition of the solutions should be tested to determine the actual percentages used. While this is difficult to account for, it is of interest to explore how concentrations may change over time both in our experimental approach and in the embryonic tissue loads.

It remains unknown whether the alcohols are penetrating the tissue. The concentrations of methanol and isopropanol within each embryo could be confirmed using headspace gas chromatography analysis. This could quantify the relative levels of alcohol concentration within

the tissue compared to exposure within the media (Lovely et al., 2014). Confirming the levels of the different alcohols within the tissue could direct future studies of how these alcohols are metabolized in zebrafish and what effect these metabolic processes have on cellular mechanisms that drive development.

Interestingly, isopropanol, but not methanol, resulted in defects to the craniofacial skeleton. These isopropanol dependent defects closely mirror the craniofacial defects observed in ethanol exposed embryos. In particular, *bmp4* embryos treated with ethanol display defects to the Meckel's cartilage as well as the trabeculae. Highly similar phenotypes were observed in the isopropanol-treated *bmp4* embryos suggesting a shared mechanism for the teratogenic actions of both alcohols. How both ethanol and isopropanol may impact the mechanisms of facial development will require extensive future analyses. However, the inroads made in gene-ethanol interactions will shed light on the mechanisms of gene-isopropanol interactions. Ultimately understanding the teratogenic actions of both alcohols will lead to broad therapeutic approaches that can be used on a number of teratogenic agents.

In a broader context, it is important to characterize how all small alcohols, such as methanol, ethanol, and propanol, act on developing embryos leading to the phenotypic effects we observe. If a sensitivity exists to one alcohol, there could also be sensitivity to other small alcohols. The dose response and phenotypic results shown here indicate that ethanol and isopropanol may act in a similar manner, but future experiments across a wider range of genetic backgrounds are necessary to confirm this suggestion. Once the toxicity and sensitivity to methanol and isopropanol in zebrafish have been determined, the next step will be to establish how gene-alcohol interactions yield the resulting phenotypes. Much remains unknown about how methanol and isopropanol affect zebrafish development but this work lays the foundation for future analyses.

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